Towards an *in vivo* biologically inspired nanofactory

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Nanotechnology is having a major impact on medicine and the treatment of disease, notably in imaging and targeted drug delivery. It may, however, be possible to go even further and design 'pseudo-cell' nanofactories that work with molecules already in the body to treat disease.

Current medical treatment for many diseases, including cancer and heart disease, involves drugs being manufactured in pharmaceutical factories and then administered to patients through a range of delivery mechanisms that include intravenous injections, intramuscular [**Author: is intramuscular a delivery mechanism on its own? If yes, it would help to have a second word like delivery**], timed release from implants, and oral ingestion. There is much research into targeted drug delivery to specific locations with controlled release schemes because this approach ensures that the drug goes where it is intended, reducing unwanted side effects and may be particularly important for highly localized diseases such as certain cancers (for example, prostate, breast, lung). There is, however, a more radical approach that draws its inspiration from the ability of the human body to self-medicate by actively adapting molecular production in response to its intrinsic biochemistry. This new approach proposes that molecular machinery could, in principle, be introduced into the body to convert pre-existing materials into therapeutic compounds, or to change molecules that a patient is unable to process, owing to some medical condition, into other compounds that the body can process. In this article we discuss the potential applications of nanotechnology to human health and the life sciences, and then propose a concept for a 'pseudo-cell factory' that might enable a high-impact new approach to medicine.

Nanotechnology research has already produced many technologies with potential medical applications that exploit the novel properties of [**Author: OK?**] quantum dots, fullerenes and carbon nanotubes, proteomic nanotechnologies, gold nanoshells, liposomes, self-assembled monolayers, nanoelectromechanical systems, and nanocomposites. Quantum dots, carbon nanotubes that emit near-infrared radiation and gold-based nanoparticles that absorb at these wavelengths have potential for both *in vivo* sensing and imaging[1], but these technologies may have limitations because, depending on their chemical composition or preparation, they have been shown to be toxic[2,3]. Self-assembled monolayers have been studied by chemists and have been used to manipulate biological responses[4]. Non-titanium orthopaedic implants comprising crystalline hydroxyapatite and peptide/hydroxyapatite are examples of nanostructured composite biomaterials[5]. These different approaches have led to numerous patents and clinical trials (for example, AmBisome, DOXIL[6]).

Drug delivery as described above is an area where nanotechnology has already had a significant impact[7]. In one ideal mode for delivery, the drug compound is: (i) encapsulated in a delivery agent;

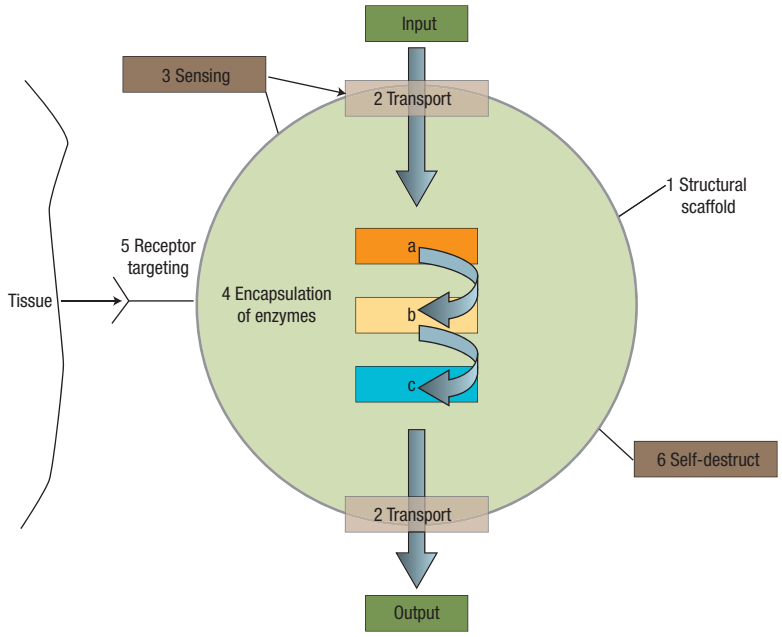

Figure 1 Schematic of six mechanisms in the biologically-inspired nanofactory: (1) a structural shell or scaffold, (2) transport to convey biomolecules to and from the environment, (3) sensing functionality, (4) encapsulation of biochemical machinery, (5) targeting of the factory within the body, and (6) externally triggered degradation to terminate a treatment in a controlled fashion.

(ii) introduced into the human body with minimal discomfort; (iii) transported to a specific location avoiding damage to the surrounding tissue or organs; and (iv) released in the specified location with a controlled concentration–time delivery profile. Packaging, release and coatings can be varied to control the introduction of drugs into the body [**Author: OK?**]. Daily or multiple injections, transdermal skin patch release and nanomicroneedle [**Author: how can a needle be nano and micro?**] delivery are mechanisms that have also been used in the past. Although these modes of delivery have been used for decades, there is still a need for refinement, to minimize irritation, as well as other improvements. For example, how can we develop new technologies to deliver unstable drugs or compounds over a specific time frame and within their window of stability? How could we develop non-invasive technologies for continuous intravenous drug treatments? How could we accurately meter real-time dosage?

Examples of nanotechnology-enabled materials for drug delivery include nanoparticle therapies using liposomes to specifically encapsulate anti-cancer drugs (for example, Abraxane, doxorubicin) and sponge-like nano-particles created with docetaxel[8]. Some other inventive approaches in this area include work by Ferrari and Desai, who have shown that porous capsules made of inorganic materials that encapsulate cells can be used to produce and release biomolecules with the cell as the production unit[9,10]. Hubbell has also demonstrated that colloidal nanomaterials could have applications in drug delivery[11]. More information on the applications of nanotechnology in biology and medicine can be found in some recent reviews[12–15].

However, instead of delivering the drug itself, could we deliver biochemical machinery to sense the need and then produce the drug on-site? We were presented with this Grand Challenge in the context of a working group of scientists, engineers and medical researchers at the National Academies Keck Futures Initiative conference in November, 2004. We have divided the challenge of designing such a biologically inspired factory into six modular tasks based on the essential components required to generate artificial cells, or 'pseudo cells', that would mimic the manufacturing capability of living cells.

We explain the requirements for each task, present examples of general approaches as potential solutions, and discuss challenges such as the immunological response and the integration of the components into a working concept. We do not give a final version of a technology that is ready to be commercially used. Rather we propose a conceptual integrated architecture based on current and future potential technology that will, we hope, highlight the challenges that must be overcome by scientists in many fields — including biology, chemistry, engineering and physics — to achieve this goal.

INSIDE THE NANOFACTORY

Our proposal is to use naturally available molecules *in vivo* and convert them into active therapeutics by designing pseudo-cell factories. These small-scale factories would provide the ability to respond to a localized health threat by targeting the appropriate region of the body, then manufacturing and delivering a biological product to treat the medical condition. A pseudo cell could produce the agent over an extended period of time and, if appropriate, be designed to target specific receptors at specific regions of the body. Here, we outline the six essential components for realizing a biochemical/biomaterials-based pseudo-cell factory: (1) a structural shell or scaffold; (2) transport to convey biomolecules to and from the environment; (3) sensing functionality; (4) encapsulation of biochemical machinery; (5) targeting of the factory within the body; and (6) externally triggered degradation to terminate a treatment in a controlled fashion (Fig. 1).

The concept of a biologically-inspired factory that can use raw ingredients native to its surroundings to produce new materials provides a new direction for future nanotechnology research, although the use of existing biological systems to produce nanoscale organic structures (for example, recombinant proteins and DNA fragments) and non-native chemical compounds is already commonplace in molecular biology. The history of artificial cell research is a long one, which can be traced back to Aleksandr Oparin in the 1920s. The artificial cell concept has included developing systems that mimic living cells as well as using cells in an artificial environment or device. Efforts to mimic live cells focus on how to generate vesicles with cellular functionality[16]. Such structures would be considered 'living' if they can replicate, self-heal, and evolve[17].

When comparing the use of an artificial cell system with living cells, there are benefits and risks to both. Living cells are wonderfully adapted to performing a wide variety of tasks. Yet, the ability to engineer control of a cell

to produce billions of molecules for a specific function has limitations. Rather than reverse engineering an extremely complex system, generating an artificial cell provides a robust platform where researchers can add functionality in a component-by-component process. Furthermore, artificial approaches may be more controllable as living cells can respond in unanticipated ways (for example, cancerous responses to treatments observed in several recent cellular therapies) introducing additional challenges. Advances in the field of artificial cells include Chang's pioneering study on animal cell encapsulation by polymer membrane capsules ('polymeric artificial cells') to produce a desired compound by using a closed-shell semi-permeable membrane shielded from the human immune system (Fig. 2)[18]. **[Author: Are changes to this paragraph OK?]**

The barriers to using artificial structures as an actual medical treatment, however, include non-standard protocols for cell encapsulation, non-uniform capsules, low biocompatibility of capsule material and safety[18]. At present, there exists a wide array of precursor biological nanostructures that could be used as pseudo-cell components. Hence, our primary focus is on synthetic structures that mimic the chemical modifying abilities of cells for *in vivo* therapeutics manufacturing.

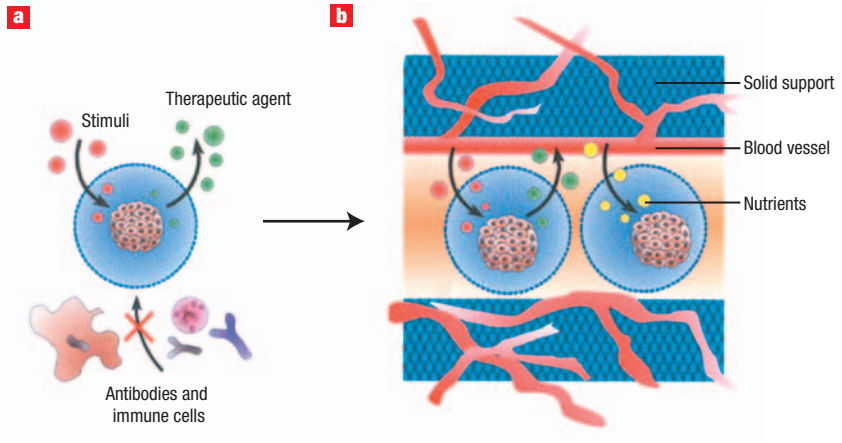

Figure 2 Schematic of the encapsulation of cells[18]. **a**, Artificial systems could allow stimuli and nutrients to pass across the membrane, while blocking the entry of antibodies or immune cells. **b**, This type of approach would enable the encapsulated cells to receive nutrition from, for example, blood vessels.

STRUCTURAL SHELL OR SCAFFOLD

Designing the shell structure is the critical first step in formulating the biologically inspired factory. There are currently a variety of techniques for this at the nano- and microscales. There are several classes of encapsulation systems: lipid-based vesicles or liposomes, diblock copolymer vesicles, nanoparticle-coated emulsions, and nanoparticle/polymer composites. Vesicles are composed of lipid surfactant molecules (Fig. 3) that assemble through hydrophobic and steric interactions to form a closed-shell bilayer[19]. The shell wall is very thin (twice the molecular length of the surfactant molecule, ~ 2 nm) with a hydrophobic interior. Liposome vesicles have been extensively studied for encapsulation and drug delivery because their wall structure is similar to the lipid bilayer wall of mammalian cells. They can also be modified to release drugs slowly and increase the therapeutic efficacy. However, liposomes can be unstable and can be taken up by the reticuloendothelial system, for example in the liver, spleen, kidney and lungs, leading to toxicity issues.

Hybrid shell structures can also be formed by merging organic and inorganic components, providing better mechanical properties while maintaining flexibility in chemistry. Such hollow spheres are relatively new and could provide interesting advantages over the all-organic shell structures for pseudo-cell applications. Two examples are layer-by-layer assembled capsules and nanoparticle-assembled capsules. Layer-by-layer capsules are prepared from a sequential deposition of polyelectrolyte and oppositely-charged nanoparticles around a spherical core[20]. This preparation method provides nanometre-level control of the composition and shell thickness; drawbacks of the method include the serial (and hence time-consuming) nature of the processing and difficulties in encapsulating target compounds. Nanoparticle-assembled capsules are prepared by combining a polyelectrolyte (such as poly-L-lysine) and EDTA to form ionically crosslinked polymer aggregates. Charged nanoparticles (such as 10-nm silicon oxide or hydroxyapatite) then adsorb around these polymer aggregates to form a larger shell-like particle[21]. These structures have thick semi-permeable walls and have the advantages that they can be prepared quickly in water at room temperature, encapsulate target compounds without damaging them, and be hollow.

At larger length scales, polymers can be synthesized with amphiphilic properties that allow self-assembly into hollow spherical structures. Recent examples include polymersomes[22] and stiff-coil copolymers[23]. Such polymeric vesicles, ranging in size from tens of nanometres to micrometres, are more resistant to mechanically or flow-induced damage. The stability of these emulsions can be greatly improved by crosslinking the nanoparticles into a continuous network, as demonstrated by Weitz and co-workers for capsules[24]. Overall, there are several options for shell structure material in the *in vivo* biologically inspired factory system. The technologies for a shell structure are currently the most developed of the six components.

An important aspect of the shell structure that will largely determine the fate of the pseudo cell *in vivo* is its ability to evade rapid clearance from circulation by the immune system, the first challenge being to avoid the innate system (for example, phagocytosis by

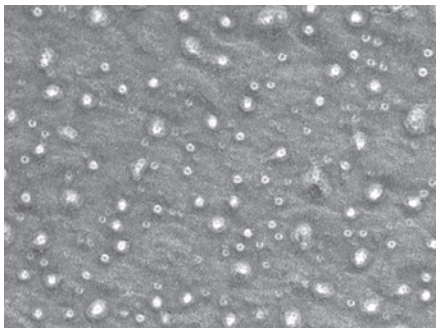

Figure 3 Lipid bilayers (liposomes) formed by thin lipid film organization. These liposomes are one potential enabling technology for creating an encapsulated aqueous environment for the *in vivo* biologically inspired nanofactory.

macrophages, natural killer cells, or complement-mediated processes). Many approaches to modifying nanoparticles for this purpose have been studied, including grafting glycosaminoglycans, glycosylphosphatidylinositols, cytokine receptors, and molecules known to resist protein adsorption such as polyethyleneglycol. These treatments have yielded limited success for retaining nanoparticles in circulation *in vivo* as the surface of any native cell is a complex one comprising a variety of glycosylated proteins and lipids.

In designing the exposed surface of a shell, it may be advantageous to mimic the surface, shape and mechanical properties of red blood cells, which remain in circulation for many days and avoid detection by phagocytes until the end of their life cycle. Suppressing an adaptive response mediated by T and B cells will be a secondary concern if clearance by the innate systems is rapid (< 48 h). It is important to note that the challenges involved in suppressing a host's immune response towards any artificial structures, such as those proposed here, are ones that also face many other existing and proposed cell-based therapies including organ transplants and stem-cell therapies.

TRANSPORT TO CONVEY BIOMOLECULES TO AND FROM THE ENVIRONMENT

[Author: is it possible to shorten this title to fit one line?] There must be a mechanism to facilitate the movement of reactants into and products out of the nanofactory. An artificial nanofactory must control the entry or exit of molecules through its encapsulating layer. This is one of the most challenging aspects of the approach, as the transport across membranes is non-trivial. Biological cells closely regulate the transport of materials across their boundaries (that is, the cell membrane) through a variety of complex mechanisms including channels, endocytosis, and lipid rafts. There is no obvious solution to this challenge at present, but research into synthetic approaches to cellular-based transport provides some insight[25]. Potential solutions include the development of active artificial carrier proteins, or the use of passive channels that allow transport across the membrane.

An active approach would involve a carrier protein. This would be inserted into the artificial membrane and would actively recognize the molecule at the exterior surface of the membrane. After binding to the carrier protein, the molecule would be transported across the membrane and released into the interior of the artificial cell. There are numerous examples of this strategy, one of which uses a transmembrane protein called GLUT1 to transport molecules including D-glucose and nicotinamide across the cell membrane[26]. By modifying carrier proteins to recognize a molecule of interest, the transport into the artificial nanofactory of this molecule could be controlled.

A second transport mechanism would entail passive transport through molecular openings designed to allow diffusion of molecules across the membrane; engineering of protein pores could enable advances in this direction[27,28]. A candidate molecule for this is the pore-forming protein α-haemolysin, which could be introduced into the lipid bilayer of the vesicles to create portals across the membrane. α-haemolysin, a toxin secreted by *Staphylococcus aureus* as a monomeric polypeptide of 293 amino acids, forms heptameric mushroom-shaped pores with a known three-dimensional structure of lipid bilayers[29]. This structure contains a 2 nm channel that spans the bilayer, and would allow diffusion of molecules of a predetermined size and charge (Fig. 4)[30]. Transport channels could be created by incorporating these molecules into the artificial membrane if the thickness of the vesicle wall is compatible with the length of the pore-forming portion of the protein.

Another passive approach is to create trans-membrane nanotubes through which chemicals can be transported. For example, carbon nanotubes with 1–2 nm pore sizes have been shown to transport water molecules rapidly[31]. Transport into and out of the artificial cell could potentially be accomplished with either one or a combination of these methods, but this will be an extremely challenging task.

SENSING FUNCTIONALITY

Having the ability to initiate transport into the pseudo cell is a prerequisite for active control of the constituents inside the factory. Regulated movement through the membranes can be controlled in cellular systems by transmembrane proteins, channels, and pumps; these are also potential directions for the artificial-cell sensing mechanism. An example of a regulation scheme that controls transport is changing the ion-conducting channels. By manipulating the amino acid sequence along the pore and helix orientation, the electrostatic and steric interactions can be altered[32], thereby controlling molecular transport through the artificial membrane.

Beyond the previously discussed idea of carrier proteins, there are other approaches as well. Membrane-spanning channels, which are made of cyclic peptides forming flat ring structures, have the ability to gate the transport of molecules across the membrane. These structures are stacked to form cylindrical nanotubes; altering the tube diameter then enables size-specific molecular transport at scales ranging from protons to glucose[33]. Another example of a regulation scheme that could be used is ligand-gated channels, which open when the target molecule binds for transport across the membrane. This approach will only be successful, however, if molecules can be detected *in vivo*.

It should be noted that although sensing of the presence or absence of molecules in applications such as drug delivery *in vivo* has been successful, there is a dearth of effective approaches for the *in vivo* sensing of biological moieties. However, the response of the pseudo cell will not be based on concentration-dictated feedback, instead the cell will sense the essential presence or absence of the target molecule and respond to this. **[Author:OK?]**

ENCAPSULATION OF BIOCHEMICAL MACHINERY

The encapsulation of molecules within the pseudo cell will enhance reaction probabilities and efficiencies owing to spatial confinement of the synthesis machinery. When a naturally occurring biochemical enters the pseudo cell, it will likely interact with encapsulated modifiers such as enzymes. By confining these modifying chemicals within a defined small area, the probability of the reaction occurring increases significantly. Furthermore, an enzymatic 'assembly line' could be created to alter the incoming molecules or 'manufacture' the product by immobilizing multiple enzymes so that their active sites are accessible only from the interior of the system.

Significant progress has been made in this direction using vesicles as the compartment material[34]. An example of a clinically relevant condition that potentially could be treated with this approach is phenylketonuria (PKU), which is linked to a deficiency of phenylalanine hydroxylase. As a simplified model system, phenylalanine hydroxylase can be encapsulated within the system and used to convert excess phenylalanine to tyrosine within the body. Devices that encapsulate phenylalanine ammonia lyase have recently been successfully used to treat rats with conditions similar to PKU[35].

The issues discussed in relation to the formation of the structural scaffold or shell envelope (introduced in component 1) must now be expanded to consider the additional task of encapsulating the machinery[36]. Vesicle-forming amphiphiles can be created from chemically defined compounds. To produce a charged lipid vesicle with enzymes, a negatively charged amphiphile or a positively charged lipid can be implanted [**Author: implanted into what?**]. Thus the encapsulation of enzymes within a system such as this proposed biologically inspired nanofactory can potentially be accomplished through different combinations [**Author:combinations of what?**]as an extension of component one.

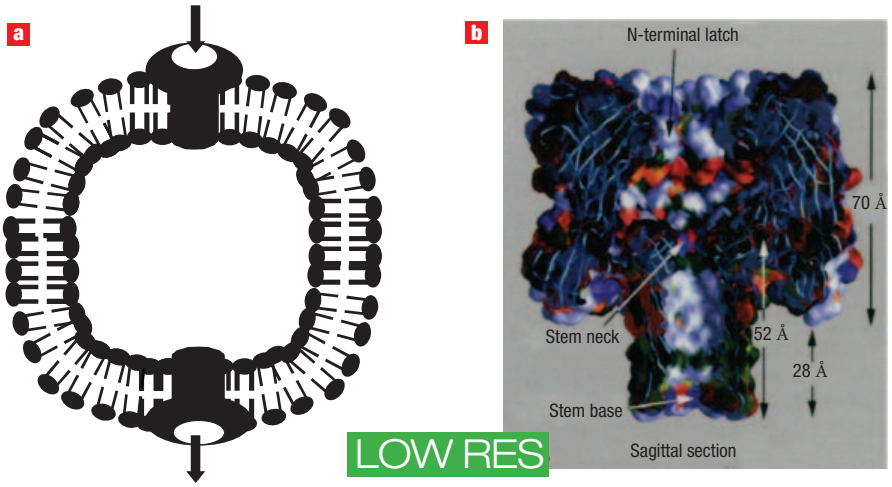

Figure 4 [Author: This caption has been edited slightly. Please check and answer the queries] One approach for the transport in an *in vivo* biological nanofactory is to create channels such as α-haemolysin that span across a membrane. **a**, Schematic showing how selected inputs **[such as?]** can enter the cell (top), while selected output **[such as?]** are able to leave (bottom). **b**, Protein structure of α-haemolysin[29]. Copyright (1996) AAAS.

TARGETING OF THE FACTORY WITHIN THE BODY

The ability to localize the factories to a specific area *in vivo* could be essential for their success. The targeting of systems on the nanoscale has already been investigated, most notably for drug delivery, and is essential for maximizing the benefits and avoiding damaging side effects, such as cell death elsewhere. Mechanisms for tissue-specific delivery of the pseudo cell could be taken from approaches that have already proved to be effective, such as receptor-ligand and antibody, which involve a molecule that will attach to the target being immobilized on the outer surface of our biologically-inspired factory. [**Author: OK?**] Receptor-ligand targeting is based on the presence of specific cell surface receptors found only in affected tissues. For example, in anti-cancer therapies, one strategy is to target dangerous cells (for example, transformed T lymphocytes) using a molecule that will attach to specific proteins (for example, interleukin-2 receptor α chain or IL-2Rα) that are expressed by these cells but not by normal T cells. [**Author: You have not actually explained what antibody targeting is. Maybe you could add in an explanation here or explain how it differs from receptor-ligand targeting**] The advantages of antibody targeting include high affinities and specificity. Fragments of antibodies offer similar advantages but will produce reduced side effects compared with whole antibodies and equivalent doses can be delivered in less volume.

Although antibodies are native agents of the immune system, their interactions with the immune system when used for targeted delivery are not entirely understood, and their use may present additional challenges in immune evasion. Further issues of biodistribution include bypassing and avoiding biological barriers that can also limit the distribution of the artificial systems. There are many biological barriers to overcome depending on the application including transdermal delivery, pulmonary delivery, oral delivery, the blood-brain barrier and peripheral nervous system [**Author: Changes to this paragraph OK?**].

These questions are being addressed in fields ranging from drug to quantum dot delivery with some successes through using biologically-inspired approaches as well as methods such as transepithelial transport pathways, receptor stimulated update and low-density porous particles[1,37]. The success of this component will require advances on many fronts.

EXTERNALLY TRIGGERED DEGRADATION TO TERMINATE A TREATMENT IN A CONTROLLED FASHION ('KILL SWITCH')

[**Author: is it possible to shorten this title to fit one line?**] Once a pseudo-cell system has been developed and delivered to specific targets *in vivo*, one critical long-term goal is to have control over their presence and activity. There must be a method to break down the system actively with a 'kill switch'. One potential idea for this feature is to take advantage of the mechanical properties of the nanofactory. With respect to systems such as lipid bilayers, forces can be imposed to overcome the molecular adhesions to break the bilayer into smaller parts, thus stopping them from functioning as a cohesive unit.

One method to disrupt spheres is to implement focused ultrasonic stimulation. A focused high-intensity ultrasonication device can be directed towards the area of the tissue where the nanofactories reside. A pulse of ultrasonic stimulation would impose a mechanical stimulation of the localized region and, by characterizing the strength of the liposomal factories, the stimulation could possibly be tuned to disrupt them alone [**Author: OK?**]. This is similar in principle to breaking up kidney stones using lithotripsy. Alternatively, the potential of using caged molecules to initiate a breakdown of the system could create an inhibitory approach.

All of these types of disruptive mechanisms have potential problems, however, including the reaggregation of the membranes to form larger structures, which might cause blockages in small capillaries. A driving goal of this component is based on the necessity to stop the production of unwanted molecules if the specific therapy is not successful or leads to unwanted side effects. Substantial efforts will need to be made to monitor side effects that occur with the production of new materials by first introducing them with conventional drug delivery or injection platforms [**Author:OK?**]. Potential clinical issues and limitations should be established far in advance of the introduction of these artificial cells so that unwanted

side effects can be detected rapidly by monitoring through urine or blood tests.

These proposed disruption components could enable spatiotemporal control of the functioning of the nanofactory *in vivo* including the ability to stop the factory when necessary [**Author: this sentence seems to repeat what has already been stated in the paragraph. Would it be ok to delete it?**].

SUMMARY

The development of an *in vivo* pseudo-cell factory could open up a new frontier in medical treatment, for example, in the treatment of phenylketonuria, which is estimated to affect approximately 1 in 20,000 live births in the United States[38], and is generally only treatable through lifelong extreme dietary restriction. We have proposed an idea that could be pursued by a diverse group of researchers as a platform technology for a variety of therapeutic approaches. This idea is centred on small-scale technologies that could produce useful products from molecules already present within the body.

Although there are many challenges that exist in developing this type of system including issues with fabrication and immunological response that would be associated with this system, if a pseudo-cell treatment were developed, this flexible approach to treating diseases *in vivo* could greatly improve the lives of many people affected by a wide variety of conditions. By combining these ideas and potential future innovations, this approach could be useful to many fields of science as well as improving human health.

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